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Research highlights

1. cDNA libraries prepared from lyophilised snake venoms.
2. Four cDNAs encoding CTLP subunits were cloned from these using a single sense primer.
3. Platelet glycoprotein Ib-binding subunit in the venom of *Agkistrodon halys* Pallas.
4. CTLP subunits within a single venom targeting coagulation factors and platelet glycoproteins.

Cloning of cDNAs and molecular characterisation of C-type lectin-like proteins from snake venoms

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Abstract

C-type lectin-like proteins (CTLPs) isolated from snake venoms are the largest and most complex non-mammalian vertebrate C-type lectin-like domain family. In the present study, we simultaneously amplified four cDNAs encoding different types of CTLP subunits from the venoms of two different species of snakes by RT-PCR with a single sense primer and a nested universal primer - two CTLP subunit-encoding cDNAs were cloned from *Deinagkistrodon acutus* venom and two from *Agkistrodon halys* Pallas venom. All four cloned CTLP subunits exhibited typical motifs in their corresponding domain regions but with relatively-low sequence similarities to each other. Compared with previously-published CTLPs, the four cloned CTLPs subunits showed slight variations in the calcium-binding sites and the disulphide bonding patterns. To our knowledge, these data constitute the first example of co-expression of CTLP platelet glycoprotein Ib-binding subunits and coagulation factors in *Agkistrodon halys* Pallas venom.

Keywords:

C-type lectin-like protein

Snake venom

RT-PCR

Agkistrodon halys Pallas

Deinagkistrodon acutus

Abbreviations:

RT-PCR, reverse transcription polymerase chain reaction; cDNA, complementary DNA; CTLPs: C-type lectin-like proteins; GP, glycoprotein; vWF, von Willebrand factor; NUP, Nested Universal Primer A ; 3'-RACE, 3'-rapid amplification of cDNA ends; Ta, annealing temperature; BLAST, Basic Local Alignment Search Tool

1. Introduction

Snake venom C-type lectin-like proteins (CTLPs) belong to the largest and most complex non-mammalian vertebrate C-type lectin-like domain family and are characterised by non-carbohydrate binding C-type lectin-like domains which make them devoid of sugar-binding abilities. CTLPs are heterodimeric structures with an α subunit of 14–15 kDa and a β subunit of 13–14 kDa and are often prone to oligomerisation to produce $(\alpha\beta)_2$ and $(\alpha\beta)_4$ (Atoda et al., 1991; Fukuda et al., 2000; Wang and Huang, 2001; Wang et al., 2003). Usually, oligomers possess platelet-activating activities by binding to von Willebrand factor (vWF) or specific glycoprotein (GP) receptors, and snake CTLPs often interact with more than one platelet receptor and have complex mechanisms of action (Clemetson et al., 2005). So far, a multitude of CTLPs with significantly-diverse bioactivities against haemostatic or thrombotic targets have been found (Clemetson et al., 2005; Morita, 2005; Ogawa et al., 2005). They primarily affect haemostasis with diverse properties, displaying different pro- or anti-coagulation effects through binding various coagulant-related factors and platelets, such as Factor IX/X, GPIb, vWF, GPVI, GPII and integrin $\alpha_2\beta_1$ receptors. The binding of the CTLPs to GP receptors or vWF that activates platelets will cause thrombosis formation and CTLPs that act in this manner are crotacetin (CTC) and convulxin (CVX) from the venom of the tropical rattlesnake, *Crotalus durissus terrificus*, that act on the collagen receptors, GPVI and GPIa/IIa (Leduc and Bon, 1998; Rádis-Baptista et al., 2006), alboaggregin-A, with 2 α and 2 β subunits, from the venom of *Trimeresurus albolabris*, that acts on GPVI and GPIb (Dörmann et al., 2001) and botrocetin and bitiscetin, derived from *Bothrops jararaca* and *Bitis arietans* venom, respectively, that initiate vWF-dependent platelet aggregation by interaction with GPIb, *in vitro* (Matsui et al., 2010). Concerning the anticoagulant CTLPs, three coagulation factor binding targets have been determined as coagulation factor IX/X (Koo et al., 2002), coagulation factor IX (Zang et al., 2003a) and coagulation factor X (Atoda et al., 1998). Other CTLPs can block the platelet aggregation pathway by inhibiting binding between vWF and the platelet GPIb-factor/IX-factor V complex, examples being agkicetin from *Agkistrodon acutus* venom and mamushigin, from *Agkistrodon halys blomohoffi* venom (Ogawa et al., 2005).

Venom from *Deinagkistrodon acutus* (the hundred-pace snake), has been well studied and a large number of bioactive proteins have been identified and characterised from this source, including

phospholipase A2, proteinase, esterase, L-amino acid oxidase, hyaluronidase and collagenase (Guo et al., 1999a; Huang et al., 1999; Zang et al., 2003b; Zha et al., 2004; Zhu et al., 2005). Heterodimeric C-type lectin-like proteins (sv-CTLs) have also been found in the venom of *Deinagkistrodon acutus*, and have been named Aall-A and Aall-B. These have an erythrocyte-targeting function rather than the traditional sv-CTL function of affecting the coagulant cascade or platelets (Xu et al., 2004). Similarly, all these enzymatic and non-enzymatic proteins, excluding CTL subunits with anticoagulation effects, have been isolated from the venom of *Agkistrodon halys* Pallas, (the Chinese water moccasin, previously named *Gloydius halys* under which name entries are referenced in the UniProtKB taxonomy data). It is also notable that the species referred to as *Agkistrodon acutus* before 1979, was renamed *Deinagkistrodon acutus* by Gloyd (Gloyd, 1979). These re-classifications to *Deinagkistrodon* and *Agkistrodon* were subsequently shown to be valid by comparative studies on skull morphology (Guo et al., 1999b). In the present study, we also attempt to address the difference between *Deinagkistrodon* and *Agkistrodon* from the perspective of venom protein composition.

The diversity and abundance of snake venom CTLs with a broad spectrum of remarkable biological activities on platelets, plasma and vessel walls, are intriguing and undoubtedly isolation and characterisation of structurally- and functionally-novel CTLs will provide more basic knowledge of their structure/function relationships and may provide the basis for drug development. As an important element of snake venom research, study of genetic mechanisms of creating functional molecular diversity can provide valuable insights into venom compositions and modes of action when combined with parallel studies in venom proteomics and physiomics. Thus, here we have used a “shotgun” cloning approach on cDNA libraries made non-invasively from venom rather than dissected venom glands, to identify and characterise cDNAs encoding different CTLs from venoms of the snakes, *Deinagkistrodon acutus* and *Agkistrodon halys* Pallas, respectively.

2. Materials and methods

2.1. Biological specimens

Crude venom was obtained from wild specimens of *Agkistrodon halys* Pallas, inhabiting Central Zhejiang Province in China and was provided by YiWu Snake Farming and Developing Company (Zhejiang, China). Venom from *Deinagkistrodon acutus* was likewise obtained from wild

specimens inhabiting Fujian Province in China. All crude venoms were lyophilised immediately after collection and maintained at -20°C prior to study.

2.2. Isolation of mRNA and cloning of CTLP-encoding cDNAs

Five milligrams of lyophilised crude venom were dissolved in 1 ml of cell lysis/mRNA stabilisation solution. By using magnetic oligo(dT) beads, according to the method described by the manufacturer (DynaL Biotech, UK), polyadenylated mRNA was isolated from the venom and its reverse-transcription products were subsequently subjected to 3'-rapid amplification of cDNA ends (RACE) procedures to acquire CTLP-encoding nucleic acid sequences using a SMART-RACE kit essentially as described by Clontech Laboratories, Inc. UK. A Nested Universal Primer A (NUP) supplied with the kit and a sense primer (5'-TGG ATT GTC CTT CTG ATT GGT CCT-3'), designed on the basis of the consensus sequences in GenBank database encoding the N-terminus of C-type lectin domains of diverse CTLP subunits, was employed in the 3'-RACE reactions. The 3'-RACE-PCR-ready cocktails, containing 0.2µl aliquots of BD (50 X) Advantage™ 2 Polymerase Mix, were committed to the gradient PCR using thermal cycler (Gene Cycler, BIORAD Hercules, CA), programmed for an initial denaturation at 94°C for 1 min, followed by 40 cycles of, denaturation at 94°C for 30s, primer annealing for 30 s at 63°C and 61°C, respectively, extension for 180s at 72°C and 1 cycle of final extension at 72°C for 10min. The resultant cDNAs were subjected to gel-analysis with controls and those with appropriate gel-bands were purified using Rapid PCR Purification Systems (Marligen Biosciences, Inc.) in accordance with the Rapid PCR purification protocol. Ligation of the purified PCR products into plasmids, was carried out overnight with pGEM-T Easy Vector (50ng) (Promega, USA), T4 DNA Ligase (3 Weiss units/µl) and 2X Rapid Ligation Buffer having a composition of 60mM Tris-HCl (pH 7.8), 20mM MgCl₂, 20mM DTT, 2mM ATP and 10% PEG, and then the resulting ligation products were transformed into *E.coli* competent cells (Stratage, USA) by the heat-shock method. Blue/white screening was performed and white colonies from each ligation were selected to isolate recombinant plasmid DNA. PCR with M13F (20 µM) forward and M13R (20 µM) reverse primer was used to confirm the existence of inserts in plasmids. In order to avoid mutants generated during PCR, six to eight recombinant plasmid DNAs for each ligation were used to conduct DNA sequence determination by using the BigDye terminator cycle sequencing mix (Applied Biosystems, USA) on an ABI 3100 automated capillary DNA sequencer (Genetic Analyzer, Applied Biosystems).

2.3. Sequence analysis

The DNA sequence obtained in ATGC format was interrogated with Chromas software (Version 1.45) and sequence proximal to the site, -AATTCGAT, which belongs to the vector pGEM[®]-T, was removed. The remaining nucleotide sequence was translated into amino acid sequence in all possible six reading frames by ExPASy (www.expasy.org/tools/dna.html).

One sequence from three or more identical clones was used as the representative for further investigation. The amino acid sequences of open-reading frames (ORF) and nucleotide sequences were compared to GENBANK databases in the National Centre for Biotechnology Information (NCBI) (www.ncbi.nih.gov) portal by using the BLAST search. Multiple alignments of nucleotide sequences and deduced amino acid sequences were performed with Vector NTI Advance[®] software and the ClustalW programme, respectively (Thompson et al., 1994) (www.ebi.ac.uk/clustalw). Protein domains were predicted with UniProt (www.uniprot.org/blast/uniprot). The phylogenetic tree constructed from the alignment was generated by a neighbour-joining algorithm (Saitou and Nei, 1987) in Lasergene 8.0 software (DNASTAR, Inc., Madison, WI, USA).

The novel sequences reported in this work were deposited in the EMBL Nucleotide Sequence Database under the following accession numbers: AhpCTLTP-1 (GenBank ID: HE804182), AhpCTLTP-2 (GenBank ID: HE804181) and DaCTLTP-1 (GenBank ID: HE804183).

3. Results and Discussion

3.1. RT-PCR amplification of CTLTPs

In an effort to extend our knowledge of CTLTPs in different snake venoms, we used the venoms from species of two, closely-related genera, *Deinagkistrodon* and *Agkistrodon*, which both belong to the Crotalinae subfamily but which had once been included in the genera, *Agkistrodon* and *Glodyius*, respectively (Gloyd, 1979). Extraction of polyadenylated mRNA for cDNA library construction was performed using lyophilised snake venoms instead of dissected venom glands, thus sparing the lives of the donor specimens. This approach was developed several years ago in our research group (Chen et al., 2002; Chen et al., 2003). The first stranded cDNAs were used as the templates in 3'RACE reactions with a designed sense primer and a nested universal primer (NUP).

Figure 1 is a gel-electropherogram of 3'RACE PCR products of cDNAs amplified using two different primer annealing temperatures (61 and 63°C) from *Deinagkistrodon acutus* and

Agkistrodon halys Pallas venom cDNA libraries, respectively, and both display a similar distribution of product bands. The bands generated using the two different PCR annealing temperatures from each cDNA library, each consist of approximately ~600-700 base pairs and are bright indicating relatively large quantities of respective products. All nucleotide sequences were double-checked by both sequence and raw data files using the ExPASy SIB Bioinformatics Resource Portal. Only cDNA sequences with at least three or more identical replicates were subjected to further processing as a means of validating real nucleotide sequences existing in the targeted snake venoms rather than coming from stochastic errors in the cloning process.

Sequence similarity searches against the GenBank database using the BLAST programme, demonstrated that all queried sequences of 519-bp from *Agkistrodon halys* Pallas venom and 542-bp from *Deinagkistrodon acutus* venom, exhibited a high identity with those encoding either coagulation factor-binding proteins or platelet glycoprotein-binding proteins.

One group of cDNAs from *Agkistrodon halys* Pallas venom, encoded a protein that binds to coagulation factors, IX or Xa, and we thus named this, AhpCTLP-1, accordingly. The predicted protein products from another cDNAs from *Agkistrodon halys* Pallas venom, encoded platelet glycoprotein Ib-binding protein and was named, AhpCTLP-2. The cDNAs from *Deinagkistrodon acutus* encoded factor Xa-binding-protein-B and agkicetin beta subunit (GPIb binding). Therefore, these corresponding deduced proteins were named, DaCTLP-1 and DaCTLP-2, respectively.

As far as we know, this is the first report of concurrent cloning of cDNAs encoding CTLPs subunits with distinct targeted molecule sites, from a single species of snake (*Agkistrodon halys* Pallas) venom using this “shotgun” cloning approach. Interestingly, AhpCTLP-1 is a coagulation factor-binding protein exhibiting antithrombotic functions, while AhpCTLP-2 acts on a platelet glycoprotein as a bidirectional modulator of platelet aggregation. Until now, there have been no reports concerning individual CTLPs that can act simultaneously with coagulation factors and platelet receptors, though structure-activity studies have indicated that CTLPs often affect more than one platelet receptor with complex mechanisms of action (Clemetson et al., 2005). Furthermore, it might also suggest that the nucleotide sequences encoding the N-terminal regions of different C-type lectin domains are conserved, which are quite different from the signal peptide gene and 5′/3′ UTRs usually reported to be highly-conserved in other components of snake venom glands, such as the snake venom metalloproteinases (SVMPs) (Selistre de Araujo and Ownby, 1995)

and phospholipase A2 (Jia et al., 2008).

In querying the entries for *Agkistrodon halys* Pallas in the Protein Knowledgebase (UniProtKB), (<http://ebi2.uniprot.org/uniprot/?query=Agkistrodon+halys+Pallas+&sort=score>), we found only 18 proteins, including phospholipases A2, L-amino acid oxidases, thrombin-like enzymes and bradykinin-potentiating peptides. However, in our laboratory, cDNAs encoding serine proteinases, metalloproteinases and other CTLP subunits (data not shown), have been cloned by RT-PCR from *Agkistrodon halys* Pallas venom. This illustrates the fact that *Agkistrodon halys* Pallas venom deserves to be further investigated.

3.2. Similarity analysis of CTLP sequences

As mentioned above, the outcomes of BLAST analyses using the GenBank database, revealed hits of 71-99% identity with many deposited/published CTLPs for each queried nucleotide sequence. AhpCTLP-1 showed 98%, 93% and 89% identities with factor IX binding protein (partial A chain) from *Gloydius halys* (GenBank ID: AY356354.1), factor Xa inhibition protein from the Korean snake (*Agkistrodon halys*) venom (GenBank ID: AF190827.1) and C-type lectin 2 from a library of *Crotalus oreganus helleri* (Southern Pacific rattlesnake) venom gland (GenBank ID: JF895761.1), respectively. AhpCTLP-2 was found to be 98% and 84% identical with mamushigin, a platelet GPIb-binding protein from *Agkistrodon halys blomhoffii* venom (Sakurai et al., 1998) and agkicetin beta subunit precursor (GPIbB2) from *Deinagkistrodon acutus* venom (GenBank ID: AF102902.1) (Chen et al., 2000).

To date, only factor IX-binding protein, belonging to the CTLP superfamily, has been reported from the venom of *Agkistrodon halys* Pallas (Zang et al., 2003a). Therefore, to the best of our knowledge, AhpCTLP-2 is the first platelet glycoprotein Ib-binding protein identified in *Agkistrodon halys* Pallas venom. DaCTLP-1 shows 95 and 94% identities to the agkisasin-b from *Deinagkistrodon acutus* venom (GenBank ID: AF350324.1) and anticoagulant protein-B (AaACP; factor Xa-binding) from the same source (GenBank ID: AB036881.1) (Tani et al., 2002), respectively. DaCTLP-2 exhibits a high similarity (99% identity) to agkicetin beta subunit precursor (GPIbB2) from *Deinagkistrodon acutus* venom (GenBank ID: AF102902.1) and B chain of antithrombin A from the same source (GenBank ID: AY091755.1). Considering that the differences exists in the primer-binding region of the nucleotide sequence, DaCTLP-2 and agkicetin beta

subunit precursors could be considered identical.

The results of BLAST analysis using the Protein Knowledgebase (UniProtKB) and multiple alignments, also demonstrated that the deduced amino acid sequences of the cloned cDNAs, share high degrees of sequence similarities to other deposited or previously-published subunits of the CTLP superfamily (Figure 2). AhpCTLTP-1 has less than 4% conflict sequences (Lys-15, Glu-24, Val-57 and Val-59) with 121-mer coagulation factor IX-binding protein (IX/X-bp) subunit A from Chinese water moccasin (*Agkistrodon halys*) venom (UniProt ID: Q7SZV0), which forms a heterodimeric CTLP with subunit B of IX/X-bp. AhpCTLTP-2 showed more than 97% similarity to mamushigin subunit beta 123 amino acid residue-domain from *Gloydius blomhoffii* (mamushi) (*Agkistrodon halys blomhoffii*) venom (UniProt ID: Q9YI92), which binds to platelet GPIb and exhibits platelet aggregation facilitation at low-shear stress and inhibition at high-shear stress. Glu-25, Gly-36 and Asp-46 are the conflict sequences compared with the ORF amino acid sequences of mamushigin subunit beta with the exception of the cleaved signal peptide consisting of the N-terminal 23 amino acid residues. DaCTLTP-1 is 99% identical with the anticoagulant protein subunit B (agkisasin-b) domain (123-mer) (UniProt ID: Q9DEF8) that possesses antithrombotic and thrombolytic activities through binding to the Gla domain of factor X and platelet GPIb- α in the presence of calcium, and blocking vWF (Mizuno et al., 2001; Tani et al., 2002). DaCTLTP-2 has the same 123 amino acid sequence, exclusive of Ser-4, as the agkicetin-C subunit- β domain from *Deinagkistrodon acutus* venom (UniProt ID: Q9DEA1), which is a potent GPIb antagonist inhibiting the vWF interaction with GPIba (Chen et al., 2000; Xu et al., 2005a; Xu et al., 2005b).

3.3 Amino acid structure analysis

The ORFs of the cloned CTLP subunit-encoding cDNAs were recognised by InterPro protein sequence analysis and classification system as 23 residues of signal peptide followed by a domain-encoding chain. Eight amino acid residue sequences immediately following the signal regions were found to vary; “DCPSGWSS”, “DCPSDWSS” and “DCPPDWSS”, which seems not to be correlated with domain composition. In this study, the corresponding nucleotide sequences of “DCPSGWSS” were used to design the sense primer. The comparison of the amino acid sequences showed that the four deduced CTLP subunits from two different species of snake venoms, exhibited a low degree of similarity. It indicated that the sequence, “DCPSGWSS” at the amino-terminus, has

a degree of conservation, as well as that distinct CTLP subunits are co-expressed in a single species of snake venom which might produce synergistic effects on different prey species. This might be an ecological adaptation to prey species. Ser-41 and Glu-47 are conserved calcium-binding site residues for all CTLP subunits. However, Glu-43 and Glu-128 in AhpCTLP-1 and Gln-43, Glu-120 in DaCTLP-1, are additional calcium-binding sites, respectively. In AhpCTLP-2 and DaCTLP-2, these additional calcium-binding sites could not be recognised previously. Cys2-Cys13, Cys30-Cys127 and Cys102-Cys119 disulphide bonds, are found in AhpCTLP-1, coupled with Cys-79, that forms an interchain bond. The remaining three CTLPs possess disulphide bonds at Cys2-Cys13, Cys30-Cys119 and Cys96-Cys111, respectively, together with Cys-75 at the interchain forming site.

3.4. Phylogenetic study of CTLPs

Molecular phylogeny has been increasingly used as a robust analytical framework for obtaining knowledge of the possible evolutionary classification of a variety of snake venom proteins. The four deduced amino acid sequences in this work, combined with eight selected CTLPs subunits from the GenBank database, were subjected to phylogenetic analysis using one of the simpler algorithms based on Distance-Tree construction. Interestingly, the grouping of these 12 CTLP subunits based on this phylogenetic analysis, coincides exactly with that of the discrete species of origin and mechanism of action through targeted molecular sites.

Group 1 consists of AhpCTLP-1, halyxin-A, helleri-B and Gh-IX-A, which all interfere with haemostasis through binding to coagulation factor IX/X. Of those, helleri-B is derived from *Crotalus oreganus helleri* (Southern Pacific rattlesnake) venom gland which differs from the other three CTLPs subunits in Group1 that are from the *Agkistrodon halys* species, thus the branch length, as shown in the phylogenetic tree (Figure 3), is longer than the other three. The 98% and 93% sequence similarities between AhpCTLP-1 and Gh-IX-A, AhpCTLP-1 and halyxin-A, respectively, also coincide with the results of phylogenetic analysis. Group 2 is comprised of DaCTLP-1, agkisasin-B and Acp-B, all from *Deinagkistrodon acutus* venom, and all possess the ability of coagulation factor-binding. Therefore, Group 2 was found to be closer to Group 1 in the phylogeny than was Group 3, which has an identical origin in *Agkistrodon halys* species, as has Group 1. Group 3 contains AhpCTLP-2 and Mamushigin-B as platelet GPIb-binding proteins. The last group,

Group 4, includes DaCTLP-2, agkicetin-B and antithrombin-AB derived from *Deinagkistrodon acutu* venom, that all show high sequence similarity (99% identity) and all affect glycoprotein I.

To summarise, we have cloned four cDNAs that encode CTLP subunits, including AhpCTLP-1 and AhpCTLP-2 from *Agkistrodon halys* Pallas venom and DaCTLP-1 and DaCTLP-2 from *Deinagkistrodon acutus* venom, by RT-PCR using a single sense primer. All four deduced CTLP subunits contain typical CTLP domains, each displaying relatively low degrees of sequence similarities to one another. In comparison with deposited and previously-published CTLP sequences, the four deduced CTLP subunits showed slight variations in the sequences of calcium-binding sites and disulphide bond locations. Most importantly, this report contains the first evidence of co-expression within the same venom, of CTLP subunits possessing the putative ability to interact with distinct molecular targets, platelet GPIb and coagulation factors, respectively. These data were generated by PCR cloning of snake venom-derived cDNA libraries using a specific sense primer and a nested universal primer. As far as we know, these data also represent the first report of cloned cDNAs encoding platelet glycoprotein Ib-binding protein from *Agkistrodon halys* Pallas venom. The successful cloning of the cDNAs encoding the CTLP subunits from *Agkistrodon halys* Pallas venom, lays a good foundation for further studies using this molecular approach on the venom of this snake. The deduced CTLP subunit structures also provide valuable and unique insights for studies on the distinctive structure–function requirements and mechanisms of action of CTLPs, in addition.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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Legends to Figures

Fig. 1

Gel-electropherogram of 3'RACE PCR products of cDNAs encoding CTLP subunits from *Deinagkistrodon acutus* (DA-) and *Agkistrodon halys* Pallas (AHP-) venom using two different annealing temperatures. S and C represent the 3'RACE PCR products and the control samples without 3'-RACE-Ready cDNA added, respectively; Subscript 1 refers to the annealing temperature of 61°C, and subscript 2, at 63°C; L, Ladder.

Fig. 2

Multiple alignment of cloned cDNA-deduced amino acid sequences of CTLP subunits obtained in this study with those of the top hits following BLASTn analysis. The alignment was generated using the ClustalW2 multiple sequence alignment programme. The different domains of proteins are indicated by arrows. The calcium-binding sites are single-underlined. Abbreviations: halyxin-A refers to halyxin A-chain precursor from *Gloydius halys*; helleri-B, helleri C-type lectin 2 from *Crotalus oreganus*; Gh-IX-A, factor IX binding protein A chain from *Gloydius halys*; mamushigin-B represents mamushigin B-chain from *Agkistrodon blomhoffii*; Agkisasin-B and Acp-B represents agkisasin-b chain from *Deinagkistrodon acutus* and anticoagulant protein-B from *Deinagkistrodon acutus*, respectively; Agkicetin-B and Antithrombin-AB represent agkicetin beta subunit precursor (GPIbB2) from *Deinagkistrodon acutus* and antithrombin A B-chain from *Deinagkistrodon acutus*, respectively.

Fig.3

Phenogram of phylogenetic relationships using the straight branch format, of CTLPs subunits predicted from their multiple amino acid sequence alignment using the ClustalW Slow-Accurate Method (Thompson et al., 1994) with a protein weight matrix: Gonnet 250. Analysis by the Kimura distance formula was used to classify these sequences into four distinct groups. The length of each pair of branches represents the distance between sequence pairs, while the units at the bottom of the tree represent the percent amino acid divergence for each group or sequence. AhpCTLP-1, AhpCTLP-2, DaCTLP-1 and DaCTLP-2 sequences were generated in the present study and the others were selected from previously-published papers. Abbreviations referring to Fig.2

LASERGENE SOFTWARE (DNASTAR Inc. Madison, Wisconsin) using the CLUSTAL W method.

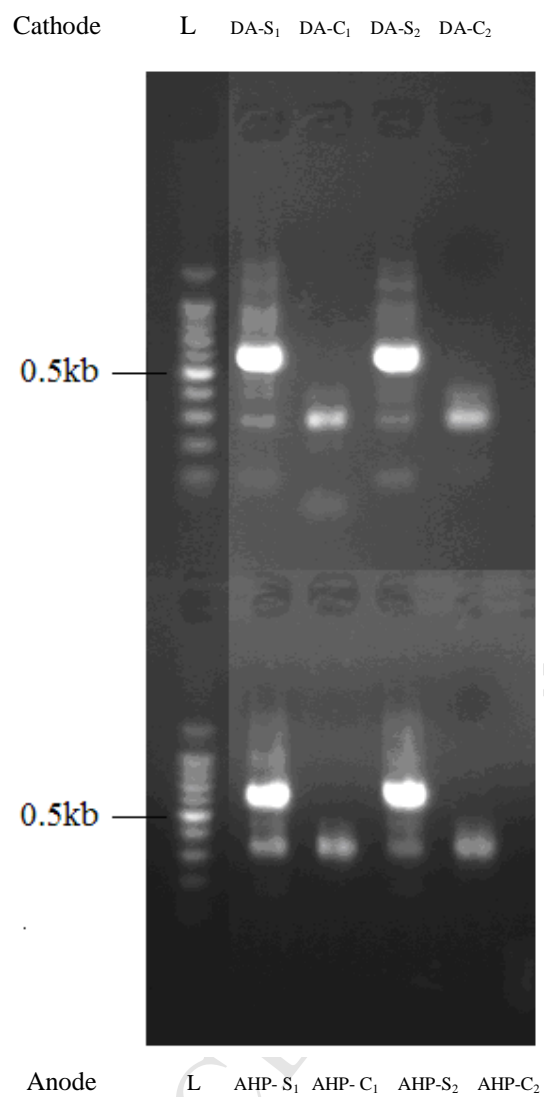
Fig.1

Fig.2

	→ Signal Peptides ←	Domain	
halyxin-A	MGRFIFLSFGLLVVFLSLSGTGADCP	SGWSSYEGHCYNIFHLFKTWAEAE	RCRKQVKG AHLVSI ESSE EADF 73
helleri-B	MGRLVFVSFGLLVVFLSLSGTGADCP	SGWSSYEGHCYQIFHVYKTWDDAE	RFCTEQAKGGHLASIESAGEADF 73
AhpCTLP-1	-----DCPSDWSSYEGHCYKTFKL	FKTWEDAESFCTEQAKGGHLVSI	ESDGEADF 50
Gh-IX-A	-----YEGHCYQTFKLFKTWADAES	FCTEQAKGGHLVSI	ESDGEADF 42
AhpCTLP-2	-----DCPSDWSSYEGHCYRVFQKEM	TWEEAEKFCTQQRKGSHLVSFHSSD	EVDF 50
Mamushigin-B	MGRFIFLSFGLLVFVFLSLSGTGADCP	SDWSSYEGHCYRVFQKEMTWEDA	EAEKFCTQQRKESHLVSFHSSEEVDF 73
Agkisasin-B	-----MDCPSDWSSYEGHCYKPFNE	LKNWADAENFCTQQHTGSHLVSFQ	STEEADF 51
Acp-B	MGRFIFVSFGLLVFLSLSGTAADCP	SDWSSYEGHCYKPFNEPKNWADA	ENFCTQQHTGSHLVSFQSTEEADF 73
DaCTLP-1	-----DCPSDWSSYEGHCYKPFNE	PKNWADAENFCTQQHTGGHLVSFQ	STEESDF 50
Agkicetin-B	MGRFIFVSFGLLVVFLSLSGTGADCP	PPDWSSYEGNCYL VVKEKKTWAE	AQKFCTEQRKECHLVSFHSAEEVDF 73
Antithrombin-AB	MGRFIFVSFGLLVVFLSLSGTGADCP	PDWSSYEGNCYL VVKEKKTWAE	AQKFCTEQRKECHLVSFHSAEEVDF 73
DaCTLP-2	-----DCPSDWSSYEGNCYL VVKE	KKTWAE AQKFCTEQRKECHLVSF	HSAEEVDF 50
		Domain	
halyxin-A	VAQLVSENMKRYGIYIWIGLRVRG	KKKQCSSQWSGSSVSYQNWIEA	ESKTCLGLQKETEF RKWFNIYCGERNPFVCEA 152
helleri-B	VAQLVANNVRKGISYIWIGLRVQ	GKEKQCSTKWSGSSVSYENWIEA	ESKTCLGLEQDTN-HKWNVIYCGQINPFVCKA 151
AhpCTLP-1	VAQLVAVNVQKTEIYVWGLRVQ	GKEQQCSSEWSGSSVSYQNWIEA	ESKTCLGLQKETEF RKWFNIYCGERNPFVCEA 129
Gh-IX-A	VAQLVAENIQKTEIYVWGLRVQ	GKEQQCSSEWSGSSVSYQNWIEA	ESKTCLGLQKETEF RKWFNIYCGERNPFVCEA 121
AhpCTLP-2	VVSMTWPILKYDFVWIGLNNIWN	ECMVETDGT RL SHNAWITESE	CIAAKTTDNQWLSRPCSRTYNV VCKFQE 123
Mamushigin-B	VVSMTWPILKYDFVWIGLNNIWN	ECMVETDGT RL SHNAWITESE	CIAAKTTDNQWLSRPCSRTYNV VCKFQE 146
Agkisasin-B	VVKLAFQTFDYGIFWMGLSNIWN	QCWNQWSNAAMLKYTDWAEESY	CVYFKSTNNKWSITCRM IANFVCEFQA 124
Acp-B	VVKLAFQTFDYGIFWMGLSKIWN	QCWNQWSNAAMLKYTDWAEESY	CVYFKSTNNKWSITCRM IANFVCEFQA 146
DaCTLP-1	VVKLAFQTFDYGIFWMGLSKIWN	QCWNQWSNAAMLKYTDWAEESY	CVYFKSTNNKWSITCRM IANFVCEFQA 123
Agkicetin-B	VVSKTFPILSYDLVWIGLNNIWN	DCMLEWSDGTKLTYKAWSGIPE	CIISKTS DNQWLSRACSR TQPFVCKFQA 146
Antithrombin-AB	VVSKTFPILSYDLVWIGLNNIWN	DCMLEWSDGTKLTYKAWSGIPE	CIISKTS DNQWLSRACSR TQPFVCKFQA 146
DaCTLP-2	VVSKTFPILSYDLVWIGLNNIWN	DCMLEWSDGTKLTYKAWSGIPE	CIISKTS DNQWLSRACSR TQPFVCKFQA 123

Fig.3